Peer Review File

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Reviewer A

You have prepared a well written manuscript for original research about the ARHGEF16 expression correlation in colon cancer cells. Below is my recommendation for revision.

1) expand briefly on your statistical analysis.

Reply: We expanded the "2.9. Statistical analysis" section.

Changes in the text: Page 8, line 155~158.

"Data were visualised by the software GraghPad prism 8.0 (GraphPad Software, USA) and statistically analysed by SPSS 24.0 (Chicago, USA). Differences were considered significant when $P<0.05$. Data are shown as mean ± standard deviation (SD). Bioinformatics analysis was performed using the R package." → "Data are shown as mean ± standard deviation (SD) and analyzed using statistical analysis software including GraphPad Prism 8.0 software (GraphPad), SPSS 24.0 (Chicago, USA) and the R package (V.3.3.4). Student's t-tests or one-way analysis of variance (ANOVA) for normally distributed data were applied to evaluate the difference between two groups or multiple groups. All experiments were carried out at least three times in triplicate. Differences were considered significant when $P<0.05$.".

2) Define and provide full form of COAD

Reply: We added the full form of COAD at its first appearance.

Changes in the text: Page 3, line 34. "colon cancer" → "colon cancer (COAD)"

3) create a table with summary of your results (3.1 to 3.4) with p values.

Reply 1: We added the table with summary of results (3.1 to 3.4).

A summary of the results (3.1 to 3.4)

Catalog	Results	P value
3.1	ARHGEF16 was highly expressed in a variety of tumours compared to normal tissues.	$P<0.05$
3.1	ARHGEF16 was highly expressed in COAD (TCGA database).	$P<0.05$
3.1	ARHGEF16 protein expression was higher in colon cancer	$P<0.05$

	tissues than in normal paracancerous tissues.	
3.2	ARHGEF16 was highly expressed in COAD cell lines.	P<0.05
3.2	We established models of SW480 and HCT116 cell lines overexpressing ARHGEF16. Meanwhile, LOVO and SW620 cell line models that silently expressed ARHGEF16 were established.	P<0.05
3.3	ARHGEF16 expression could affect the proliferative ability of colon cancer cells, with high expression of ARHGEF16 promoting the proliferation and growth of colon cancer cells and, conversely, low expression of ARHGEF16 reducing the proliferation and growth of colon cancer cells.	P<0.05
3.4	Expression of ARHGEF16 was closely associated with the migration and invasive ability of colon cancer cells, and that overexpression of ARHGEF16 could promote the migration and invasion of colon cancer cells and correlate with the metastatic potential of colon cancer.	P<0.05

Reviewer B

This is an interesting study. However substantial information is missing from the Materials and Methods. Also, some methods used to generate data have not been included in the Methods section. Please see below.

Sample collection from Patients

Please provide information on this. Were samples collected at surgical resection? What was the approximate size of the sample? Was the sample collected from the tumour leading edge and from an area distant to the tumour? Were samples snap frozen in liquid nitrogen or placed in RNA later?

Reply: (1) Samples collected at surgical resection. (2) The approximate size of the sample was 0.5cm×0.5cm×0.5cm. (3) Paracancerous sample collected from the tumour leading edge area. (4) All fresh tissues were transferred to liquid nitrogen immediately after acquisition.

Changes in the text: Page 6, line 99 and 100.

"Cancerous and paracancerous tissue (control group) specimens were collected from six operated colon cancer patients and all tissues were stored at -80°C." → "Six pair of tumor/paracancerous

samples collected at surgical resection. The approximate size of the sample was 0.5cm×0.5cm×0.5cm. Paracancerous sample collected from the tumour leading edge area. All fresh tissues were transferred to liquid nitrogen immediately after acquisition. "

Cell Culture:

It's not clear how cell viability was determined. Was a manual or automated hemocytometer used? Were cell viability counts performed in triplicate?

Reply: The cell viability was determined by an Automated Cell Counter (Bio-Rad, USA). The cell viability counts performed in triplicate.

Changes in the text: Page 6, line 110. We add the following sentence at the end of the line 110. "The cell viability was determined by an Automated Cell Counter (Bio-Rad, USA). The cell viability counts performed in triplicate.".

Western analysis:

Primary and secondary antibodies are not listed; antibody source not supplied; concentrations of primary and secondary antibodies not included. What detection method does the imaging system use; what were the exposure times, etc.

Reply: We added the necessary information according to your comment in "2.4. Protein blotting analysis" section, including the name, source and dilution ratio of primary and secondary antibodies; and the detection method.

Changes in the text:

Changes in the text: Page 6, line 117, 118 and 119. "Primary antibodies were incubated overnight at 4 °C and secondary antibodies were incubated after elution." → "Primary antibodies ARHGEF16 (ab154785, abcam, United Kingdom) with a 1:2000 dilution and GAPDH (ab9485, abcam, United Kingdom) with a 1:5000 dilution were incubated overnight at 4 °C and a horseradish peroxidase-conjugated secondary antibody (GB23303, Servicebio, China) with a 1:3000 dilution was incubated after elution.".

Changes in the text: Page 6, line 119 and 120. "The data were analysed using an Odyssey infrared imaging system and its own software." → "Protein bands were visualized using an enhanced chemiluminescence (ECL) kit (Beyotime, China). The data were analyzed using an Odyssey infrared imaging system and its own software with an exposure time 10 s."

RT-PCR:

How was RNA quality and quantity determined after extraction?

What instrument was used for real time PCR?

What primer design software was used for the primer design and where were the primers

synthesized?

Reply: We added the necessary information according to your comment in “2.5. qRT-PCR” section, including RNA's quality and quantity determination, a real time PCR instrument and primer design software.

Changes in the text:

Changes in the text: Page 6, line 124. We add the following sentence at the beginning of the line 124. "The quality of RNA was determined by agarose gel electrophoresis assay, and the concentration of RNA was detected by NanoDrop (Thermo Scientific, USA)."

Changes in the text: Page 6, line 125. "q-PCR was performed using TB Green reagent" → “q-PCR was performed using a 7500 Real-Time PCR System and TB Green reagent".

Changes in the text: Page 6, line 129. "Primer sequence" → “Primers were designed by Primer Premier 5 (Premier Biosoft, Canada) and synthesized by Sangon Biotech (Shanghai, China). Primer sequence".

Cell proliferation assay

There is no information on the transfection procedure- the product used, the transfection efficiency, the plasmid used for overexpression etc.

Some basic information should be provided on how the cell counting kit works. How many replicates were carried out? Was a standard error of the mean calculated?

Reply 1: We added “Lentiviral transfection” into the “2.3. Cell culture” section concerning transfection and some basic information about cell proliferation assay.

Changes in the text: Page 6, line 111. We add the following sentence in line 111. “COAD cell lines were transfected with a lentiviral vector to regulate the expression of ARHGEF16. The lentiviral vector was designed and constructed by GeneChem Co. Ltd. (Shanghai, China). Third-generation COAD cell lines were seeded on 96-well plates at a density of 1×10^4 cells/ml. When the cell confluence rate approached 1/3, transfection was carried out according to the instructions.”

Changes in the text: Page 7, line 138. “The values were recorded for each plate.” → “The values were recorded for each plate 5 times.”

Scratch assay

There is minimal information for this assay. Were non-transfected cells used as a control?

How were cells viewed and imaged? After the scratch, were cells rinsed to remove debri? Were multiple measurement points taken within the cell-free gaps? How was Image J used for the analysis?

Reply: (1) There was no transfected cells used as a control in this study, but we set up an empty vector as control group. (2) We chose appropriate time points for taking photos (0h and 24h), and

placed the cells under an inverted microscope to obtain pictures. (3) We wash the cells with PBS 3 times before each photo session to fully wash away the residue. (4) We taken 6 measurement points within the cell-free gaps. (5) Image J used for the analysis with the migration index=migration distance of test group/migratance distance of control group.

Changes in the text: Page 7, line 142~144. “After transfection, when the cells had grown to 90% confluence, they were scratched by applying a 200 μL gun tip, photographed at 0 and 48 h and analysed by applying Image J software.” → “After transfection, when the cells had grown to 90% confluence, they were scratched by applying a 200 μL gun tip. Following wash the cells with PBS 3 times before each photo session to fully wash away the residue, we photographed at 0 and 48 h under an inverted microscope and analysed by applying Image J software with the migration index=migration distance of test group/migratance distance of control group.”.

Transwell invasion assay

Where were the transwell chambers sourced from? Which basement membrane matrix was used and how much? Was the gentian violet extracted from the matrix? What microscope was used and at what magnification were cells counted? Were non-transfected cells used as a control? How many replicates were carried out?

Reply: We added the necessary information according to your comment in “2.8. Transwell experiment” section, including the details of Transwell chambers, Matrigel matrix and 0.1% Crystal Violet Ammonium Oxalate Solution.

Changes in the text:

Changes in the text: Page 8, line 147. "Transwell chambers with basement gel" → "Transwell chambers (Corning, USA) with 80 μL Matrigel matrix (Corning, USA) ".

Changes in the text: Page 8, line 151. "gentian violet" → "Crystal Violet Ammonium Oxalate Solution".

Changes in the text: Page 8, line 152. "a microscope" → "a inverted microscope (×200). The cells were counted in triplicate assays.".

Gene knockdown

There is no information on the siRNA mediated knockdown of ARHGEF16. How was the siRNA designed and where was it synthesized? What controls were used? How was the siRNA administered? How many replicates were performed?

Reply: We added “Lentiviral transfection” into the “2.3. Cell culture” section concerning transfection and some basic information about cell proliferation assay.

Changes in the text: Page 6, line 111. We add the following sentence in line 111. “COAD cell lines were transfected with a lentiviral vector to regulate the expression of ARHGEF16. The lentiviral

vector was designed and constructed by GeneChem Co. Ltd. (Shanghai, China). Third-generation COAD cell lines were seeded on 96-well plates at a density of 1×10^4 cells/ml. When the cell confluence rate approached 1/3, transfection was carried out according to the instructions."

Colony formation and CCK-8 Assay

No information provided for colony formation and the CCK8 assay.

Reply: Detailed information on CCK-8 can be found in line 134~138. In addition, we have added the information for colony formation.

Changes in the text: Page 7, line 139.

We add the following sentence in line 139. "For colony formation assay, briefly, 400 COAD cells with over-expressed or under-expressed ARHGEF16 were seeded into a 6-well plate. And cells were maintained until colonies formed. Colonies were then fixed by paraformaldehyde for 30 min and stained using crystal violet. The colonies were then calculated.".